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(54) Title: A PROCESS OF CELL REPROGRAMMING THROUGH PRODUCTION OF A HETEROKARYON

(57) Abstract

The invention is directed to reprogramming an adult cell nucleus to produce an embryo. The process involves: taking a donor cell or nucleus; fusing the donor nucleus with a recipient cell that has not been enucleated to produce an aneuploid cell; waiting a period of time to allow the donor nucleus to be reprogrammed; and removing the recipient cell nucleus. This process results in an embryo with a reprogrammed nucleus.

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A PROCESS OF CELL REPROGRAMMING THROUGH PRODUCTION OF A HETEROKARYON

Field of invention

The present invention relates to the production of animals, in particular ungulates using nuclear transfer or cloning technology, and animals so produced. More specifically, the invention relates to nuclear transfer, nuclear transfer embryos, and animals so produced.

Background of the invention

Nuclear transfer involves insertion of a donor cell or nucleus (karyoplast) into an enucleated oocyte (cytoplast) and reprogramming of the donor nucleus by the recipient cytoplasm. In all published protocols enucleation is carried out at the beginning of the process. In general, nuclear transfer protocols include:

1) activation of a recipient oocyte;
2) enucleation of the chromosomes from the recipient oocyte; and
3) transfer of donor nucleus to the enucleated oocyte.

It is generally believed that enucleation of the recipient oocyte is necessary and maintains ploidy (correct chromosome number) following the insertion of the donor nucleus (USPN 5,453,366; USPN 6,011,197; USPN 5,945,577; USPN 5,994,619 and USPN 5,843,754).

Embryo cloning using enucleated oocytes have been successfully performed in a variety of animals such as cattle (Kanka *et al* (1991) *Mol Reprod Dev* 29, 110), sheep (Wilmut *et al* (1997) *Nature* 385, 810), rabbits (Collas and Robl (1991) *Biol Reprod* 45, 455), and pigs (Prather *et a.* (1990) *J Exptal Zool* 255, 355).

Enucleation may be achieved by physical or other means. The original procedure described for sheep and cattle involved bisecting the oocyte to produce two cytoplasts, one of which did not contain chromosomes (Willadsen *Nature* (1986)320 63). This procedure has a number of disadvantages chief amongst which is the physical damage done to the oocyte and the reduction in the volume of cytoplasm both of which reduce development of embryos produced therefrom.

More recently a different procedure has been used to enucleate oocytes which is less invasive and removes the chromosomes with a minimum of cytoplasm. In this method the first polar body and a small amount of the adjacent cytoplasm is aspirated to remove the metaphase plate maternal chromosomes. This is done in conjunction with a DNA specific flourochrome (Hoechst 33342) to ensure that the maternal chromosomes are removed from the oocyte (Tsunoda *et al* (1988) *J Reprod Fert* 82 173 and PCT Publications 97/07668 97/07669). Alternatively, enucleation can be achieved by functional enucleation such as application of ultraviolet radiation or other nucleus inactivating method. Transfer of donor nucleus immediately or shortly following enucleation is currently the preferred method for livestock species (PCT Publication 97/07668). Although the physical damage is reduced the problem acutely remains that development to term of embryos so produced is very poor (around 1% for sheep and cattle embryos constructed using embryonic cells (Prather & First (1990) *J Reprod Fertil suppl* 41 125).

There is a distinct need for methods of nuclear transfer which give rise to embryos with increased capacity to develop to term in host animals.

Summary of the Invention

In a first aspect this invention relates to a nuclear transfer method involving non enucleation or enucleation following transfer of a donor cell or karyoplast into a recipient cytoplast. This method results in the production of a triploid embryo initially, which when not enucleated has the potential to revert to a diploid state following expulsion of the recipient pronucleus at the first cleavage or thereafter. Alternatively the recipient pronucleus (and any retained polar bodies) are enucleated following nuclear transfer typically prior to the first cleavage.

Thus, there is provided in a first aspect of this invention, a process of nuclear transfer comprising providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the recipient to form an NT embryo, optionally removing cytoplast chromosomes, and optionally thereafter culturing the NT embryo to

allow one or more cell divisions.

In an embodiment of this first aspect of the invention there is provided a process of nuclear transfer comprising providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the recipient cytoplast to form an NT embryo, and optionally thereafter culturing the NT embryo to allow one or more cell divisions.

In a second embodiment of the first aspect of the invention, there is provided a process of nuclear transfer comprising providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the recipient cytoplast to form an NT embryo, subsequently removing cytoplast chromosomes from the NT embryo, and optionally thereafter culturing the NT embryo to allow one or more cell divisions. Thus enucleation takes place post fusion according to this embodiment.

Transfer of the donor cell or karyoplast into the recipient cytoplast containing the recipient chromosomes may be effected by a number of different methods including membrane fusion, or direct injection of the karyoplast into the recipient cytoplast. At the time of karyoplast insertion, the reconstructed embryo, which may be referred to as an "NT embryo", may be activated by physical or chemical means. Alternatively, activation may take place before or following insertion of the donor nucleus.

After cleavage, the NT embryo can be bisected at any suitable stage, (for example, at the 2-32 cell stage) using physical or chemical means (embryo splitting). Embryonic cells or blastomeres may be isolated therefrom and used in second and subsequent rounds of nuclear transfer to provide multiple NT embryos capable of development to term (serial cloning), to give a plurality of NT embryos capable of subsequent division and development.

In another aspect of the invention, a karyoplast may be recovered from an NT embryo following one or more divisions, and used for a second round of nuclear transfer by

transfer into a second recipient cytoplast to improve nuclear reprogramming. A second round of nuclear transfer has been used to increase the developmental competence of mouse NT embryos (Kwon & Kono (1996) *Proc Natl Acad Sci* USA 93 13010). The second cytoplast can be an oocyte, zygote or any other embryo. The second cytoplast may be enucleated prior to the insertion of the karyoplast, or non enucleated or enucleated post fusion according to the present invention.

In another aspect of the invention there is provided a process for the clonal generation of an animal which process includes providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the recipient to form an NT embryo, optionally removing cytoplast chromosomes, and optionally thereafter culturing the NT embryo to allow one or more cell divisions. Optionally cells or nuclei from the NT embryo can be used as karyoplasts in a second round of nuclear transfer to improve nuclear reprogramming. Thereafter one or more NT embryos are transferred into a recipient female whose oestrous cycle has been appropriately synchronised. Development through pregnancy gives rise to one or more cloned off-spring.

Detailed description of invention

Nuclear transfer involves insertion of a donor cell or nucleus (karyoplast) into an enucleated oocyte (cytoplast), with subsequent reprogramming of the donor nucleus by the recipient cytoplasm.

Uses for nuclear transfer or cloning technology include: the production of large numbers of genetically identical or similar animals or clones from an individual animal for purposes of animal breeding; the production of genetically manipulated i.e. transgenic animals in which extra genetic information has been inserted or existing genetic information deleted (gene knockout); and the dedifferentiation of somatic cells to produce a population of pluripotent cells which can then be differentiated to cells, tissues or organs for the purpose of cell therapy, gene therapy, organ transplantation etc. Such cells have an advantage in that they can be autologous ie obtained initially from the patient and as such are not destroyed by the patient's immune system.

Prior to the present invention, procedures for cloning mammals required enucleation of the recipient cell prior to nuclear transfer. In accordance with a first aspect of this invention there is provided a process of nuclear transfer comprising providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the recipient to form a NT embryo and optionally thereafter culturing the NT rembryo to allow one or more cell divisions. Alternatively the recipient pronucleus (and any retained polar bodies) is enucleated following fusion and /or activation prior to the first cleavage, and the NT embryo is cultured to allow one or more cell divisions.

Both processes result in an increase in embryo development. For example the number of nuclear transfer embryos that develop in vitro to the blastocyst stage (see Table 1) and their cell number (both of which are important in ensuring the development of NT embryos to term) both increase.

The invention is applicable to a variety of animals including those having medical, research, commercial or other importance such as economic value. For example, the invention is applicable to economically important ungulates such as pigs, cattle, sheep, goats, water buffalo and camels as well as animals of use in scientific and medical research such as monkeys, rabbits, rats, mice, hamsters and guinea pigs. Additionally, other animal species such as rodents, birds, amphibians and fish can also be produced by the methods of the present invention. It should also be noted that the invention is applicable to other animal species of social or ecological value such as, for example, horses, llamas and domestic pets, as well as other animals such as domesticated or wild animals. In the latter case, the invention is, if desired, applicable in the propagation of endangered species or in the revival of extinct animal species.

The overall procedure disclosed herein is generally the production or cloning of an animal by nuclear transfer (NT) into a non-enucleated donor cell or prior to enucleation of a donor cell. In general, the animal will be produced by a nuclear transfer process comprising the following steps:

1) selection and isolation of nucleus from a donor cell;

2) selection and isolation of a recipient cell (generally, but not necessarily, an oocyte from an animal of the same species as the source of the donor nuclei);

3) transfer of the desired donor cell nucleus into the recipient cell cytoplasm to create a "nuclear transfer" (NT) unit;

4) activation of the recipient cell (e.g. oocyte);

5) culture of the activated NT unit to a greater than 2-cell developmental stage or to an embryo stage; and

6) transfer of the NT embryo into a host animal such that the NT embryo develops into a fetus or cloning of the NT embryo to obtain multiple embryo clones having the same genetic make up.

The present invention is equally useful in producing transgenic as well as non-transgenic animals. Transgenic animals can be produced from genetically modified donor cells. The term "transgenic" as used herein, is intended to mean any cell, tissue, embryo, fetus or animal whose DNA has undergone recombinant technology, genetic engineering or other technical intervention. For example, an animal in which one or more endogenous gene has been deleted, duplicated, modified or activated in any way is a transgenic animal. An animal may also be a transgenic animal as a result of its carrying one or more "transgene".

As used herein, the term "transgene" is intended to refer to any exogenous DNA sequence or genetic material which does not naturally form part of or does not originate from the genetic material of the animal intended to be genetically modified.

Thus, a transgenic animal can be cloned by the process herein by use of donor nucleus which has been subjected to genetic manipulation prior to its transfer into a recipient oocyte or cell. Methods by which a desired gene can be inserted, removed or modified in the donor cell nucleus are known in the art and include, for example, homologous sequence targeting (recombination) such as described in USPN 5,763,240 and Capecchi, *Science*, 244:1288 (1989); *in vitro* duplex targeting, described, for example in Sena and Zarling *Nature Genetics* 3:365 (1993); as well as more standard recombinant methods such as

site-specific mutagenesis (Carter et al., *Nucl Acids Res* 13:4331 (1986); Zoller et al. *Nucl Acids Res* 10:6487 (1987), cassette mutagenesis (Wells et al. *Gene* 34:315 (1985)), restriction selection mutagenesis (Wells et al. *Philos Trans R Soc London SerA* 317:415 (1986) or other known techniques for modifying a DNA sequence.

Thus, according to the present invention, reproduction or multiplication of mammals having specific or desired genotypes is possible. In addition, the present invention can also be used to produce animals which can be used, for example, in cell, tissue or organ transplantation, or to produce animals which express desired compounds such as therapeutic molecules, growth factors, or other medically desired peptide or protein.

Transfer of the donor cell or karyoplast into the recipient cytoplast containing its recipient chromosomes may be effected by a variety of techniques. For example, membrane fusion, direct injection of the karyoplast into the recipient cytoplast or other means to give an NT embryo. At the time of karyoplast insertion, the reconstructed embryo may be activated by physical or chemical means. Alternatively, activation may take place before or following insertion of the donor nucleus.

After cleavage, the NT embryo can be bisected at any suitable stage, (for example, at the 2-32 cell stage) using physical or chemical means (embryo splitting). Embryonic cells or blastomeres may be isolated therefrom and used in second and subsequent rounds of nuclear transfer to produce multiple NT embryos capable of development to term (serial cloning).

In another aspect of the invention, a karyoplast may be recovered from an NT embryo following one or more divisions, and used for a second round of nuclear transfer by transfer into a second recipient cytoplast to improve nuclear reprogramming. A second round of conventional nuclear transfer has been used to increase the developmental competence of mouse NT embryos (Kwon & Kono (1996) *Proc Natl Acad Sci* USA 93 13010). The second cytoplast can be an oocyte, zygote or any other embryo. The second cytoplast may be enucleated prior to the insertion of the karyoplast, or non enucleated or

enucleated post fusion according to the present invention.

Donor cells can also be embryonic cells, embryonic stem cells, primary cell cultures, cultured cell lines derived from embryonic, fetal or somatic cells, and the like. By way of further example, an embryonic cell may be a blastomere, for example a 16-32 cell mass (morula), or a pluripotent cell derived from a blastocyst. The donor cell may be subject to conventional recombinant DNA manipulation. For example, genes may be deleted, duplicated, activated or modified by gene additions, gene targeting, gene knock-outs, transgenesis with exogenous constructs which may or may not contain selectable markers may be accomplished by techniques such as microinjection, electroporation, viral-mediated transfection, lipofectin, calcium-phosphate precipitation (Lovell-Badge, *"Introduction of DNA into embryonic stem cells"* in: *Teratocarcinomers and Embryonic Stem Cells: A Practical Approach*, IRL Press, Oxford, E.J. Robertson, ed. pp 153-182, 1987; *Molecular Cloning: A Laboratory Manual*, Volume 2 & 3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Sambrook, Fritsch and Maniatis Ed. pp 15.3-15.50, 16.3-16.68, 1989). Additional techniques for transforming mammalian cells are described, for example, in Keown et al. (*Methods in Enzymology* 185:527-537, 1990) and Mansour et al. (*Nature* 336:348-352, 1988).

The donor cells may come from any animal as described previously, including livestock animals or companion animals, such as pigs, monkeys, horses, sheep, cattle, goats, dogs, and cats. A donor cell derived from an animal can be isolated from nearly any type of tissue or organ. For example, a biopsy can be taken from an animal, the cells from the biopsy sample can be separated and the cells isolated using culture techniques well known to a person of ordinary skill in the art. Suitable isolated cell types include somatic cells (differentiated or undifferentiated) and germ cells, such as for example, fibroblast cells, epithelial cells, endothelial cells, hematopoietic cells, neural cells, keratinocytes, melanocytes, chondrocytes, cardiocytes, muscle cells, embryonic cells and fetal cells. A karyoplast is a donor nucleus, or a zona damaged cell containing a nucleus, or donor cell.

Fibroblasts can be preferable because they are easily obtained (either from fetal or adult

tissue sources), can be obtained in large quantities and are easily propagated, genetically modified and cultured *in vitro*.

The recipient cytoplast can be an oocyte, zygote or any cell from an embryo. Suitable animal sources of oocytes can be as described above for sources of donor nuclei. Preferably, the oocytes are obtained from a vertebrate animal and more preferably, an ungulate. Ova or oocytes may be readily collected from the reproductive tracts of ovulating animals using surgical or non-surgical methods. Methods for isolating oocytes are well known in the art. Ovulation may be induced by administering gonadotrophins of various species origin to animals. Oocytes may be collected by aspiration from mature follicles, or collected following ovulation. Alternatively immature oocytes may be collected from the ovaries of living or slaughtered animals and matured in vitro using standard procedures such as described in WO 90/13627 ("In vitro maturation of bovine oocytes in media containing recombinant gonadotropins along with bovine oviductal cells", 1989). Oocytes can be fertilised *in vivo* or *in vitro* to yield zygotes. Where the stage of maturation of the oocyte is believed to be important or of concern to successful nuclear transfer, selected metaphase II oocytes (as determined by presence of polar bodies) can be used as recipients for nuclear transfer.

Again, the recipient cytoplast may come from any animal as described above for donor nucleus, including livestock animals or companion animals, such as pigs, horses, sheep, cattle, goats, dogs, and cats. Preferably the donor cell and recipient cell are from the same species.

The present invention eliminates the need to enucleate oocytes or allows this to be done following fusion and/or activation.

In the case where enucleation is not performed the embryo contains the maternal haploid nucleus (N) and the fibroblast nucleus (2N) i.e. is a triploid embryo. The maternal nucleus is most likely expelled with reversion to the diploid state (2N) at or soon after the embryo starts to divide (cytokenesis)

Importantly we have found that by not enucleating or enucleating following fusion and/or activation, the development of NT embryos increases. While the exact mechanism(s) involved remain to be determined, it would be readily apparent that this increase in development represents a significant improvement in methodology. Increased survival of embryos improves the whole technique of producing animals by nuclear transfer technolgoy.

In the first report on the development of nuclear transfer embryos constructed using porcine fetal fibroblasts for example (Du *et al Theriogenology* 1999 51: 201 abstract) we reported development to the blastocyst stage of 3% using oocytes enucleated prior to fusion. Whereas we have found in the present invention by not enucleating or enucleating 4-6 h post fusion/activation development to the blastocyst stage was increased by more than five fold (Table 1).

Cell fusion may be carried out by any means known in the field. Established methods for inducing cell fusion include exposure of cells to fusion-promoting chemicals, such as polyethylene glycol (see, for example, Kanka *et al*, (1991), *Mol. Reprod. Dev.*, 29, 110-116), the use of inactivated virus, such as sendi virus (see, for example, Graham *et al*, (1969), *Wistar Inst. Symp. Monogr.*, 9, 19), and the use of electrical pulses (see, for example, Willasden, (1986), *Nature*, 320, (6), 63-36 and Prather *et al*, (1987), *Biol. Reprod.*, 37, 859-866). Use of electrical stimulation or cell fusion is preferred but by no means essential to this invention. By way of example 2 to 6 electrical pulses may be delivered to the entities at an interval between each pulse of from one minute to sixty minutes, such as 2 pulses 30 minutes apart. Each pulse may be in the form of a set of pulses, such as 2 to 4 pulses, spread from each other by 1 to 20 seconds. DC pulses are generally used at a voltage such as 150v/mm for a duration such as 60μs, and generally with a pre- and post-pulse alternating current.

Alternatively a donor nucleus (karyoplast) can be isolated from a cell and injected directly into the cytoplasm of the recipient cytoplast. Direct micro injection of a karyoplast into a

donor cell may be carried out by conventional method, such as disclosed by Wakayama *et al* (1998) *Nature* 394, 369-374.

Activation is carried out by standard procedures including by physical means such as electrical pulses, chemical means such as use of calcium ionophore or other means. Activation can done at the same time as fusion (simultaneous fusion and activation) or subsequently (fusion then activation subsequently). Allowing the nucleus to spend time in the recipient cytoplasm prior to activation can be beneficial in terms of reprogramming and subsequent development.

NT embryos can be cultured in vitro for one or more divisions. After cleavage, the NT embryo can be bisected at any suitable stage, (for example at the 2-32 cell stage) using physical or chemical means (embryo splitting). Embryonic cells or blastomeres may be isolated therefrom and used in second and subsequent rounds of nuclear transfer to produce multiple NT embryos capable of development to term (serial cloning).

Serial nuclear transfer may also be conducted. A karyoplast may be recovered from an NT embryo following one or more divisions, and used for a second round of nuclear transfer by transfer into a second recipient cytoplast to improve nuclear reprogramming. Each NT embryo can itself be used as a nuclear donor, such as, for example at the morula or 32-64 cell stage. Alternatively, inner cell mass cells from the blastocyst stage can be used as a nuclear donor as described in Zakhartchenko et al. *Molec Reprod and Develop* 44:493 (1996). Additionally, a new cell line to be used as a source of nuclear donor cells could be produced from an NT embryo produced according to the present invention.

A second round of nuclear transfer has been used to increase the developmental competence of mouse NT embryos (Kwon & Kono (1996) Proc Natl Acad Sci USA 93 13010). The second cytoplast can be an oocyte, zygote or any other embryo. The second cytoplast may be enucleated prior to the insertion of the karyoplast, or non enucleated or enucleated post fusion according to the present invention.

NT embryos can be cultured in vitro for one or more divisions to assess their viability or transferred to the reproductive tract of a recipient female, or stored frozen for subsequent use by standard procedures.

Nuclear transfer embryos can be transferred immediately after reconstruction or following a period of in vitro culture to the reproductive tract of a recipient female using standard surgical or non surgical procedures. In the case of litter bearing species such as the pig it may be advantageous to transfer one or more non-NT embryos (helper embryos) to help initiate and maintain pregnancy, and additionally assist in parturition. Alternatively nuclear transfer embryos can be encapsulated in agar to protect them from immune attack and cultured in vivo in the ligated oviduct of a temporary recipient, recovered and then re-transferred to a second recipient. Transferred NT embryos (and helper embryos) may be allowed to develop to term.

In another aspect of the invention there is provided a process for the clonal generation of an animal which includes providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the cytoplast to give an NT embryo and optionally culturing the NT embryo *in vitro* to allow one or more cell divisions to give one or more NT embryos. Thereafter one or more NT embryos are transferred into a recipient female whose oestrous cycle has been appropriately synchronised, pregnancy term giving rise at the term of pregnancy to one or more cloned off-spring.

The clonal generation of animals generally involves introducing into a recipient female animal, a plurality of embryos, comprising NT embryos with or without non NT (helper) embryos as herein described. For example, in pigs from 5 to 50 embryos may be introduced into the reproductive tract of a recipient female animal whose oestrous cycle has been appropriately synchronised using standard procedures. 1 to 3 embryos may be introduced into the reproductive tracts of synchronised sheep or cattle. The embryo may be transferred surgically or non-surgically. For example the embryo may be inserted into the uterus using an appropriate device, such as a catheter, or alternatively may be introduced into a fallopian tube for passage into the uterus, using standard procedures.

According to a further aspect there are provided genetically identical animals when prepared according to the above process.

Another aspect this invention relates to progeny of nuclear transfer animals (NT animals) produced according to this invention. Progeny result from crossing an NT animal with another animal to give offspring. The other animal may be selected for a particular trait. A progeny animal contains a part of the genetic complement of the original donor karyoplast, which can be conveniently detected, for example, by DNA markers.

According to another aspect of this invention there is provided a cloned animal produced from a nuclear transfer embryo. The present invention as described herein provides for developmentally competent nuclear transferred embryos that give rise to cloned animals. In this regard the progeny or cloned animals contain DNA that is identical to that of the karyoplast used in their production as described herein. Accordingly animals of significant agricultural fitness may be produced expressing desired beneficial traits such as low fat meat, rapid growth. Animals that have been genetically modified for example for biomedical applications such as xenotransplantation may also be produced.

A further aspect this invention relates to the use of cloned animals as herein described in agriculture, for organ production, or oocyte and embryo production. The capacity to clonally manipulate animals means that desirable characteristics can be directly exploited. By way of example, in agriculture, animals can be produced as a source of low fat meat as a result of genetic manipulation, such as homologous recombination. By such an approach, the cloned animals can be used in general for highly efficient and desirable agricultural and animal husbandry pursuits, including as a source of organs for human transplants (for example, where antigens have been removed, masked or attenuated by genetic manipulation).

The process of the present invention may include genetic manipulation of the donor cell or karyoplast prior to transfer into the recipient cytoplast. Alternatively, or in addition,

genetic manipulation may take place following NT cell production, that is genetic manipulation on the NT embryo.

Genetic modification or manipulation involves conventional recombinant DNA manipulative techniques wherein the DNA within a cell is subject to recombinant DNA technology. For example, as described previously, genes may be deleted, duplicated, activated or modified by gene additions, gene targeting, gene knock-outs, transgenesis with exogenous constructs which may or may not contain selectable markers may be accomplished by techniques such as microinjection, electroporation, viral-mediated transfection, lipofection, calcium-phosphate precipitation (Lovell-Badge, "Introduction of DNA into embryonic stem cells" in: *Teratocarcinomers and Embryonic Stem Cells: A Practical Approach*, IRL Press, Oxford, E.J. Robertson, Ed. pp 153-182; *Molecular Cloning: A Laboratory Manual*, Volumes 2 and 3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Sambrook, Fritsch and Maniatis Ed. pp 15.3-15.50, 16.3-16.68, 1989).

Genetic manipulation may confer a desirable trait on the resultant embryo and/or the animal produced therefrom. For example, an embryo (and its' resultant animal) carrying an inactivated, modified or replaced gene can be obtained using targeted homologous recombination as described, for example, in Zarling et al. USPN 5,763,240. Targeting an exogenous DNA sequence to an endogenous, predetermined DNA sequence can be achieved by:

1) assembly of the gene construct from an appropriate region of the genomic clone of the target gene from the selected animal species. If desired, introduce a specific mutation into a desired region of the gene;

2) mix the altered gene construct with an appropriate recombinase enzyme (e.g. RecA from *E. coli*);

3) inject/insert the protein-DNA mixture into a recipient oocyte by nuclear transfer;

4) transfer the oocyte containing the transgene into a host animal (preferably of the same species); and

5) breed the animal to homozygosity to achieve the desired trait.

Alternatively, the protein-DNA mixture can be transfected into a donor cell line, such as porcine fibroblasts. The targeted cells may be either proliferating (US 5,945,577) or arrested in G0/G1 phase (Wilmut *et al* 1997 385:810-813). Those cells containing the correctly targeted transgene sequence can then be used as donor cells. Nuclei from the donor cells are then used as donor nuclei for nuclear transfer to recipient oocytes.

Targeted genetic manipulation of the donor cell nuclei can be performed for a variety of reasons, such as for example, to correct a mutant gene to produce a functional gene product, to place an endogeneous or transgene under specific control (such as regulated expression using inducible or suppressable promoters), to inactivate an endogenous gene, or to replace expression of an endogenous gene with an equivalent gene from a different species.

DNA constructs used to produce a transgenic or donor cell whose nuclei has been genetically modified can also comprise a variety of elements including regulatory promoters, insulators, enhancers and repressors as well as encoding for ribozymes and anti-sense DNA or RNA, depending upon the need and desired genotype of the resultant animal or cell.

Thus, the present invention can be used to provide an animal with a specific or desired genotype and the resulting phenotype. A transgenic animal or chimeric animal expressing a therapeutic human protein or molecule can be obtained by the present invention. For example, a transgenic animal expressing a human-like hemoglobin gene can be produced. Examples of recombinant human-like hemoglobins are provided in US patents 5,028,588; 5,545,727; 5,545,727; 5,844,090; and WO 95/03820. Donor nuclei for nuclear transfer can be obtained from animal cells which have been altered to have their endogenous animal hemoglobin gene replaced with a human-like hemoglobin gene. Replacement of the endogenous animal hemoglobin gene with a human-like gene can be achieved using, for example, targeted homologous recombination as described in Zarling *et al.* USPN 5,763,240. The transgenic animal cell is now suitable as a source of donor nuclei for

nuclear transfer to produce an animal expressing human-like hemoglobin, which can then be harvested from the animal and purified to produce a pharmaceutical useful for treating patients in need of extra oxygen-carrying capacity. Methods for using pig embryonal germ cells to produce a transgenic pig are known and described, for example in PCT Publication 97/25413.

Alternatively, where desired, the invention can be applied in the nuclear transfer of embryos to produce transgenic animals expressing desired therapeutic proteins in their milk such as described in EPO 264 166, "Transgenic Animals Secreting Desired Proteins into Milk"; WO 94/19935, "Isolation of components of Interest from Milk"; and WO 95/17085 "Transgenic Production of Antibodies in Milk."

This invention can also be used to produce transgenic animals such as pigs, the organs of which are suitable for transplant into human patients in need of a heart or kidney, for example, a procedure known as "xenotransplant". In xenotransplant, a natural pig organ would be rejected by the human recipient's immune system within minutes to hours by a process known as hyperacute rejection. Hyperacute rejection occurs because all humans carry "xenoreactive antibodies" against a carbohydrate antigen on pig tissue. The antigen is galactose (alpha 1,3) galactose, also called gal epitope. When the xenotransplant recipient's xenoreactive antibodies bind the gal epitope on the transplanted pig tissue, the human complement cascade is initiated, leading to rejection and necrosis of the pig organ. In order to prevent or ameliorate hyperacute rejection of a transplanted pig organ, a transgenic pig can be produced which expresses an enzyme which reduces the level of the gal epitope, and which also produces a human complement inhibitor (WO 97/12035). Another way to prevent hyperacute rejection of a pig organ is to use a transgenic pig which does not express the endogenous enzyme which synthesizes the gal epitope, namely gal (alpha 1,3) galactosyl transferase (US patent numbers 5,849,991 and 5,821,117). Homologous recombination techniques can be used where it is desirable to "knock-out" or delete an endogenous gene. Alternatively, an endogenous gene can be inactivated such as by antisense or other recombinant techniques.

This invention will now be described with reference to the following non-limiting examples.

Examples

An example for each method is described with reference to nuclear transfer in the pig. Oocytes were collected from superovulated Large White x Landrace donor pigs 50-54 h post hCG and denuded of cumulus by pipetting and hyaluronidase treatment. Fibroblasts were obtained from day 25 fetuses and cultured in DMEM plus 10% FBS. NT embryos were cultured in NCSU23 for 7 days with 10% FBS added at 96h.

In the first example enucleation was not performed and the cells used were a random cycling population of fibroblasts derived from day 25 porcine fetuses. We and others have shown that the majority of these cells (60%) are in G0/G1. In the second example enucleation was performed 4-6 hrs after activation which occurred at the same time as fusion. Fetal fibroblasts synchronised at GO/G1using serum depletion. (Wilmut et al 1997 385 810-813)

Results using the two methods are shown in Table 1. Previous work where oocytes were enucleated prior to fusion and constructed with serum starved fetal fibroblasts is also given by way of comparison. (Du et al Theriogenology 1999 51 201).

Method 1 Non–enucleation of oocytes

The oocyte zona was pierced using a beveled pipette and an individual karyoplast inserted into the perivitelline space. The karyoplast-oocyte complexes were cultured in NCSU23 medium until activation and fusion. Fusion and activation of the karyoplast-oocyte complexes was induced using a BTX Electro Cell Manipulator ECM 2001 (BTX CA). The complexes were first washed in fusion medium containing 0.3m mannitol/100μm $CaCl_2$/ 200μm $MgSO_4$/0.01% polyvinylalcohol and then placed between two wire electrodes (1 mm apart) of the fusion chamber (450-10WG, BTX, CA) with 0.1 ml of fusion medium. Activation and membrane fusion were simultaneously simultaneous fusion

and activation) induced by applying two DC pulses (150 V/mm, 60 μs) at 5 sec intervals with a pre-, and post- pulse alternating current (AC) field of 4V, 1 MHz for 5 sec each).

Method 2 Enucleation following fusion and/or activation

The oocyte zona was pierced using a beveled pipette and an individual karyoplast was inserted into the perivitelline space of each oocyte at a location opposed to the first polar body. The karyoplast-oocyte complexes were cultured in NCSU23 medium until activation and fusion. Fusion and activation of the karyoplast-oocyte complexes was induced using a BTX Electro Cell Manipulator ECM 2001 (BTX CA). The complexes were first washed in fusion medium containing 0.3m mannitol/100μm $CaCl_2$/ 200μm $MgSO_4$/0.01% polyvinylalcohol and then placed between two wire electrodes (1 mm apart) of the fusion chamber (450-10WG, BTX, CA) with 0.1 ml of fusion medium. Activation and membrane fusion were simultaneously induced by applying two D.C. pulses (150 V/mm, 60μs) at 10 sec intervals with a pre-, and post- pulse alternating current (AC) field of 4V, 1 MHz for 5 sec each).

The oocyte derived chromatin in the form of pronuclei (plus any retained polar bodies) was removed 3-6h following fusion. NT embryos that were fused and activated simultaneously were centrifuged in the presence of a cytoskeletal inhibitor (Cytochalasin B) to polarize the cytoplasmic lipid droplets permitting visualization of nuclear structures in the cytoplasm. The nucleus nearest to the hole in the zona made during insertion of the karyoplast is the donor nucleus. The oocyte derived pronucleus located away from the karyoplast nucleus was aspirated using a beveled pipette (30-40 μm in diameter) in PB1 + 10 % Fetal Calf Serum containing 7.5 μg/ml Cytochalasin B (Sigma).

Table 1

In vitro development of non-enucleated, enucleated post fusion and NT embryos enucleated prior to fusion

NT Method	Cell	Expts	n	2-cell	4-cell	Morula	Blast	Cell No (Mean ± SEM) (range)
Non-enucleated	Unsyn	2x	26	20(77)b	16(62)c	12(46)c	11(42)c	41.0 ± 4.9b (20 –55)
Enucleated post fusion	G0	6x	100	73(73)b	44(44)b	26(26)b	18(18b)	24.7 ± 4.1a (10 –63)
Enucleated prior to fusion	G0	5x	103	58(56)a	27(26)a	7(7)a	3(3)a	26.3 ± 3.4a (22 –33)

Within columns numbers with different superscripts are significantly different ($P<0.05$)

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" or the term "includes" or variations thereof, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers. In this regard, in construing the claim scope, an embodiment where one or more features is added to any of claims is to be regarded as within the scope of the invention given that the essential features of the invention as claimed are included in such an embodiment.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process of nuclear transfer comprising providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the recipient to form an NT embryo, optionally removing cytoplast chromosomes, and optionally thereafter culturing the NT embryo to allow one or more cell divisions.

2. A process according to claim 1 wherein a karyoplast is recovered from an NT embryo, and transferred into a second recipient cytoplast containing its chromosome content, optionally removing the cytoplast chromosomes, and optionally culturing the nuclear transferred cytoplast to allow one or more cell divisions.

3. A process according to claim 1 wherein transfer of the donor cell results from cell fusion between cytoplast and donor cell.

4. A process according to claim 1 wherein activation takes place at the same time as fusion, or occurs subsequently.

5. A process according to claim 1 wherein the cytoplast chromosomes are not removed from the NT embryo.

6. A process according to claim 2 wherein the cytoplast chromosomes are removed from the NT embryo.

7. A process for the clonal generation of an animal which process includes providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the cytoplast to give an NT embryo and optionally culturing the NT embryo *in vitro* to allow one or more cell divisions to give one or more NT embryos, and thereafter transferring a plurality of NT embryos into a recipient female animal which at the conclusion of pregnancy term gives rise to one or more cloned off-spring.

8. An NT embryo produced according to any of claims 1 to 6.

9. An NT embryo according to claim 8 wherein the donor cell or karyoplast comprises a transgenic cell from an animal selected from pigs, cattle, sheep, goats, and rabbits.

10. An NT embryo according to claim 9 wherein the transgenic cell comprises a DNA sequence encoding a protein selected from a human-like hemoglobin, a human complement inhibitory factor, and an enzyme which masks or reduces the level of a xenoreactive antigen.

11. An NT embryo according to claim 8 wherein a DNA sequence encoding an endogenous protein is inactivated or deleted.

12. A process for the clonal generation of an animal which process includes providing a recipient cytoplast without removing chromosomes therefrom, transferring a donor cell or karyoplast into the cytoplast to give an NT embryo, removing the cytoplast chromosomes from the embryo, optionally culturing the NT embryo *in vitro* to allow one or more cell divisions to give a plurality of NT embryos, and thereafter transferring one or more embryos into a recipient female animal which at the conclusion of pregnancy gives rise to one or more cloned offspring.

13. An animal produced from an NT embryo produced according to any of claims 1 to 11.

14. A process according to claim 1 wherein after one or more cell divisions the NT embryo is bisected to give a plurality of NT cells capable of subsequent division and developments.

15. A process according to claim 1 wherein the donor cell or karyoplast is subject to genetic modification prior to transfer into a recipient cytoplast.

16. A process according to claim 15 wherein said genetic modification comprises inserting one or more nucleic acid sequences into the donor cell or karyoplast chromosome content.

17. A process according to claim 15 wherein said genetic modification comprises deleting one or more nucleic acid sequences from the donor cells or karyoplast chromosome content.

18. A process according to claim 14 wherein the NT embryo is subject to genetic modification.

19. The process according to claim 16 wherein the one or more nucleic acid sequence encodes a human hemoglobin.

20. A process according to claim 17 wherein the one or more nucleic acid sequence encodes an endogenous hemoglobin.

21. A process according to claim 15 wherein said genetic modification comprises deleting one or more nucleic acid sequences from an NT embryo cell chromosome content.

22. A process according to claim 15 wherein said genetic modification comprises inserting one or more nucleic acid sequences into an NT cell chromosome content.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00432

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A01K 67/00 C12N 5/16 15/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See electronic data bases

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE: "cloning, organism"/CT AND "cell nucleus: tr, transplantation"/CT
WPIDS: (nuclear OR nucleus) AND (C12N 5/06 OR C12N 5/22 OR C12N 5/16 OR C12N 15/06 OR C12N 15/07 C12N 08)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Katsutoshi Niwa et al *Development Growth and Differentiation* (1999) pp163-172 "Transplantation of blastula nuclei to non-enucleatd eggs in the medka, *Oryzias latipes*" Whole document	1 - 22

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 2000	07 JUL 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **J.H. CHAN** Telephone No : (02) 6283 2340

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00432

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos : **1 - 22 partially**

because they relate to subject matter not required to be searched by this Authority, namely:

The claims included within their scope processes for the generation of humans. The claim are directed to the production of reprogrammed animal cells, which includes reprogrammed human cells. The claims have not been searched in so far as they include reprogramming of human cells.

2. [ ] Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.